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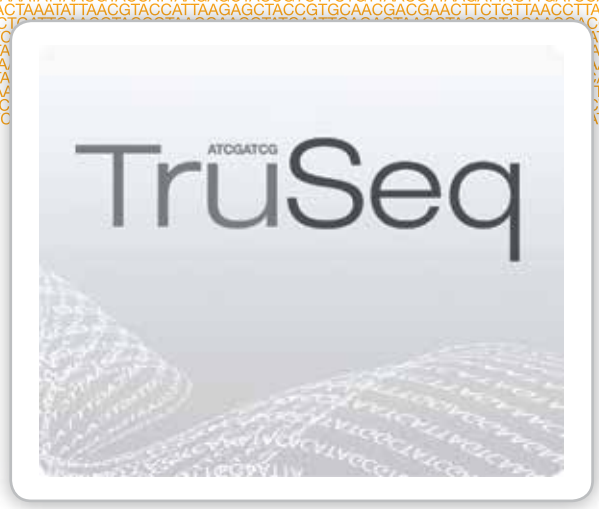
TruSeq® DNA PCR-Free Library Prep Guide



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Customize a short end-to-end workflow guide with the Custom Protocol Selector
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Revision History

Part #	Revision	Date	Description of Change
15036187	C	December 2014	• Kit names changed from 'sample' prep to 'library' prep • Added references to BaseSpace® for organizing samples, libraries, pools, and runs • Removed use of plate name (e.g., IMP plate), except for first instance and last instance in each procedure • Bead cleanup procedures modified to remove EtOH ***before*** placing plate on the magnetic stand to dry. • Added centrifuge step before each thermal cycler incubation in the LS protocol • Added well volume to heat incubation procedures • Updated *Prepare Adapter Setup* sections • Updated *Additional Resources* • Removed List of Tables • Updated SDS link to support.illumina.com/sds.html

15036187	B	November 2013	• Renamed Incubate 1 IMP to Incubate IMP
			• HS protocol
			  • Corrected Make DCT procedure to clarify that the DCT plate is an HSP plate
			  • Moved centrifuge steps after incubate
			• EUC and LTF merged into a single document per protocol
			• Created new appendix of *Supporting Information* containing *Acronyms*, *Kit Contents*, *Consumables and Equipment*, and *Indexed Adapter Sequences*
			• Replaced *Best Practices* section with a reference to content on the Illumina website
			• Replaced *Adapter Options* and *Pooling Guidelines* sections with a reference to the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*
			• Removed *Usage Guidelines*

15036187	A	January 2013	Initial Release

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Overview

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Introduction

This protocol explains how to prepare up to 96 uniquely indexed paired-end libraries of genomic DNA (gDNA) using Illumina® TruSeq® DNA PCR-Free Library Prep kits. The purpose of the protocol is to add adapter sequences onto the ends of DNA fragments to generate indexed single read or paired-end sequencing libraries. The libraries are ready for subsequent cluster generation and sequencing.

The TruSeq DNA PCR-Free Library Prep protocol offers:

- Streamlined workflow
  - Master-mixed reagents to reduce reagent containers and pipetting
  - Universal adapter for preparation of single read, paired-end, and indexing
- Optimized shearing for whole-genome resequencing with 350 bp and 550 bp insert size workflows
- Bead-based size selection reagents included in each kit
- Optimized workflows for processing low sample (LS) and high sample (HS) numbers
- Low-throughput (LT) and high-throughput (HT) kit configurations
- High throughput
  - Adapter plate allows for simultaneous preparation of 96 dual-indexed DNA samples
  - Volumes optimized for standard 96-well plate
- Advanced troubleshooting
  - Process control checks built-in for quality control
- Index adapter tags for all samples
  - Additional adapters and primers are not necessary
  - Each TruSeq DNA PCR-Free LT Library Prep Kit contains adapter index tubes recommended for preparing up to 24 samples for sequencing. Together kits A and B allow for pooling up to 24 samples
  - The TruSeq DNA PCR-Free HT Library Prep Kit contains a 96-well plate with 96 uniquely indexed adapter combinations designed for manual or automated preparation of 96 uniquely indexed samples

The protocol is compatible with no indexing or a lower indexing pooling level. The libraries generated do not require PCR amplification to enable cluster generation.

Protocol Features

This guide documents the TruSeq DNA PCR-Free Library Prep protocol using a TruSeq DNA PCR-Free LT Library Prep Kit or TruSeq DNA PCR-Free HT Library Prep Kit.

- Chapter 2 Low Sample (LS) Protocol—Explains how to perform TruSeq DNA PCR-Free Library Prep using the Low Sample Protocol
- Chapter 3 High Sample (HS) Protocol—Explains how to perform TruSeq DNA PCR-Free Library Prep using the High Sample Protocol

Equivalent results can be expected from either protocol, however the HS protocol can yield more consistent results between samples. Their distinguishing elements are as follows:

Table 1 Protocol Features

	Low Sample	High Sample
LT Kit - Number of samples processed at the same time	≤ 24 with indexed adapter tubes*	> 24 with indexed adapter tubes*
HT Kit - Number of samples processed at the same time	≤ 24 with indexed adapter plate	> 24 with indexed adapter plate
Plate Type	96-well 0.3 ml PCR 96-well midi	96-well HSP 96-well midi
Incubation Equipment	96-well thermal cycler	Microheating systems
Mixing Method	Pipetting	Microplate shaker

* Each TruSeq DNA PCR-Free LT Library Prep Kit contains enough reagents to prepare up to 24 samples. When used together, TruSeq DNA PCR-Free LT Library Prep Kits A and B allow for pooling up to 24 samples using the 12 different indexes in each kit. Illumina does not recommend preparing more than 24 samples at a time using the LS protocol. An alternative to using the HS protocol for more than 24 samples is to perform separate library preparations to ensure robust performance.

The TruSeq DNA PCR-Free Library Prep fragmentation process is optimized to obtain final libraries, with the following average insert size.

Table 2 Insert Size Options

Insert Size	350 bp	550 bp
Input DNA Per Sample	1 µg	2 µg
Recommended Read Length	≤ 2 x 101 bp	≤ 2 x 151 bp*

* Read lengths greater than 2 x 151 bp produce a significantly higher percentage of overlapping read-pairs.

DNA Input Recommendations

It is important to quantify the input DNA and assess the DNA quality before beginning TruSeq DNA PCR-Free Library Prep.

Input DNA Quantification

Follow these DNA input recommendations:

- Correct quantification of gDNA is essential.
- 1 µg input DNA is recommended for the 350 bp insert size workflow.
- 2 µg input DNA is recommended for the 550 bp insert size workflow.
- The ultimate success or failure of library preparation strongly depends on using an accurately quantified amount of input DNA.
- Use multiple methods of quantification to verify results.
- Illumina recommends using fluorometric based methods for quantification, such as Qubit or PicoGreen, to provide accurate quantification of dsDNA. UV spectrophotometric-based methods, such as Nanodrop, measure any nucleotides present in the sample including RNA, dsDNA, ssDNA, and free nucleotides. These methods can give an inaccurate measurement of gDNA.
- DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to the presence of excess nucleic acids.
  - These methods require the preparation of calibration curves and are highly sensitive to pipetting error.
  - Make sure that pipettes are correctly calibrated and are not used at the volume extremes of their performance specifications.

Assessing DNA Quality

Absorbance measurements at 260 nm are commonly used to assess DNA quality:

- The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity, and values of 1.8–2.0 are considered indicative of relatively pure DNA.
- The presence of RNA or small nucleic acid fragments, such as nucleotides, can compromise both absorbance measurements.
- Carefully collect gDNA samples to make sure that they are free of contaminants.

In-Line Control DNA

The End Repair Control, A-Tailing Control, and Ligation Control contain DNA fragments used as controls for the enzymatic activities of the End Repair Mix 2, A-Tailing Mix, and Ligation Mix 2, respectively. Each reagent contains dsDNA fragments designed to report the success or failure of a specific enzymatic activity used in the library preparation process. Sequencing determines the readout. If the sequence of an in-line control is in the final sequencing data viewed in the Sequence Analysis Viewer (SAV), it indicates that its corresponding step was successful. If it does not, or if it is in substantially diminished numbers, it indicates the step failed. The controls are intended for troubleshooting and are useful for identifying the specific mode of failure, but are uninformative in cases where sequencing data are not generated from a library.

NOTE

The use of these controls is optional and they can be replaced with the same volume of Resuspension Buffer.

The control molecules work through the design of their ends. Controls are added to the reactions before their corresponding step in the protocol. Their end structures match the end structures of a DNA molecule that has not gone through the step. If the step is successful, the control molecule is modified to participate in downstream reactions of library generation and resulting in sequencing data. If the step fails, the control molecule does not go forward in the process and no sequencing data are generated.

Table 3 In-Line Control Functions

Reagent	Function	Control	Structure of Control DNA Ends
End Repair Mix 2	End repair: Generate blunt ended fragments by 3'–> 5' exonuclease and 5'–> 3' polymerase activities	End Repair Control 1*	5' overhang at 1 end, 3' overhang at other end
End Repair Mix 2	End repair: Add 5'-phosphate groups needed for downstream ligation	End Repair Control 2*	Blunt with 5'-OH group
A-Tailing Mix	A-tailing: Make fragments compatible with adapters and prevent self-ligation by adding a 3'-A overhang	A-Tailing Control	Blunt with 5'-phosphate group
Ligation Mix 2	Ligation: Join 3'-T overhang adapters to 3'-A overhang inserts	Ligation Control	Single-base 3' 'A' base overhang

*End Repair Control 1 and End Repair Control 2 are separate controls included in the End Repair Control reagent

The control reagents can be used for various library insert sizes. Each is provided in ladders ranging from approximately 150–850 bp in 100 bp increments. Each control molecule has a unique DNA sequence, indicating both its function and size. The RTA software (v1.9, and later) recognizes these sequences and isolates the control sequences from the main body of sequencing reads. RTA reports the control sequences counts per lane in the controls tab of the RTA status.html page. The CTE1 and CTE2 controls show a narrow distribution of sizes while the CTA and CTL controls show a broad size distribution, because the size selection step is before A-Tailing. For more information regarding the control read-out in the SAV, see the *Sequence Analysis Viewer User Guide (part # 15020619)*.

Additional Resources

The following documentation is available for download from the Illumina website.

Resource	Description
TruSeq DNA PCR-Free Library Prep LS Protocol Experienced User Card (part # 15036190)	Provides LS protocol instructions, but with less detail than what is provided in this guide. **New or less experienced users are advised to follow this guide and not the Experienced User Card (EUC).**
TruSeq DNA PCR-Free Library Prep HS Protocol Experienced User Card (part # 15036191)	Provides HS protocol instructions, but with less detail than what is provided in this guide. **New or less experienced users are advised to follow this guide and not the Experienced User Card (EUC).**
Dual Index Sequencing with TruSeq HT Library Prep (part # 15059916)	Provides guidelines for preparing for dual-indexing sequencing when using a TruSeq DNA PCR-Free HT Library Prep Kit.
TruSeq Library Prep Pooling Guide (part # 15042173)	Provides TruSeq pooling guidelines for preparing libraries for Illumina sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.
Illumina Experiment Manager Guide (part # 15031335) and *IEM TruSeq DNA, RNA, or ChIP Quick Reference Card (part # 15037152)*	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
BaseSpace User Guide (part # 15044182)	Provides information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.

Visit the TruSeq DNA PCR-Free LT Library Prep Kit support page or TruSeq DNA PCR-Free HT Library Prep Kit support page on the Illumina website for access to requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Low Sample (LS) Protocol

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Introduction

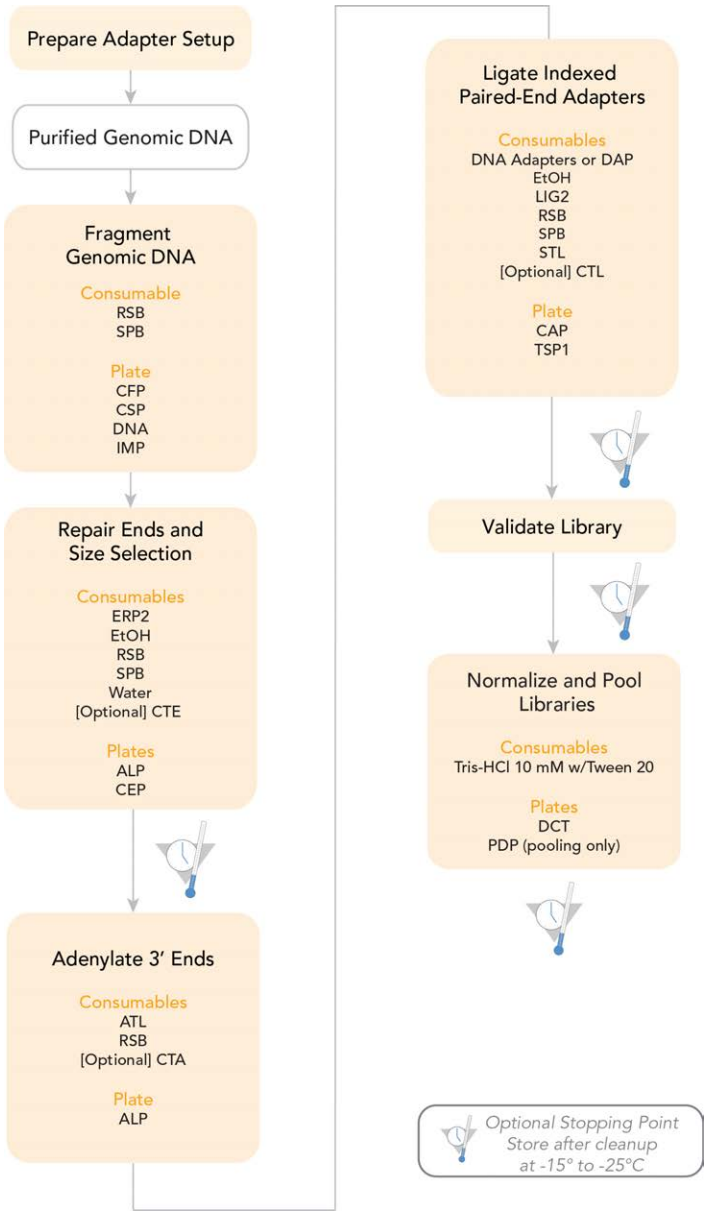
This chapter describes the TruSeq DNA PCR-Free Library Prep LS protocol. This protocol is intended for preparing up to 24 samples at the same time using either the LT or HT kit.

- Follow the protocol in the order described, using the specified volumes and incubation parameters.
- Review Best Practices before proceeding. See *Additional Resources* on page 6 for information on how to access TruSeq DNA PCR-Free Library Prep Best Practices on the Illumina website.
- Review Appendix A Supporting Information before proceeding, to confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the LS protocol.

Library Prep Workflow

The following figure illustrates theTruSeq DNA PCR-Free Library Prep LS workflow.

Figure 1 TruSeq DNA PCR-Free Library Prep LS Workflow



Prepare Adapter Setup

If you are pooling, use IEM or BaseSpace to record information about your samples before beginning library prep.

- Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software.
- Use BaseSpace to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software.

NOTE

For information about IEM and BaseSpace software documentation on the Illumina website, see *Additional Resources* on page 6.

Review the planning steps in the *TruSeq Library Prep Pooling Guide (part # 15042173)* when preparing libraries for Illumina sequencing systems that require balanced index combinations.

Fragment DNA

This process describes how to optimally fragment the gDNA depending on the Covaris platform used. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process is optimized to obtain final libraries with the following average insert sizes:

Table 4 Insert Size Options

Insert Size	350 bp	550 bp
Input DNA Per Sample	1 µg	2 µg
Recommended Read Length	≤ 2 x 101 bp	≤ 2 x 151 bp*

* Read lengths greater than 2 x 151 bp produce a significantly higher percentage of overlapping read-pairs.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C (2°C to 8°C after initial thaw)	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Barcode labels for: • CFP (Covaris Fragmentation Plate) • CSP (Clean Up Sheared DNA Plate) • DNA (DNA Plate) • IMP (Insert Modification Plate)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plates	4	15°C to 30°C	User
Covaris tubes	1 per sample	15°C to 30°C	User
DNA samples	• 1 µg per sample for a 350 bp insert size • 2 µg per sample for a 550 bp insert size	-25°C to -15°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Preparation

- Review *DNA Input Recommendations* on page 4.

- Review best practices for handling magnetic beads. See *Additional Resources* on page 6 for information about TruSeq DNA PCR-Free Library Prep best practices on the Illumina website.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the Resuspension Buffer from -25°C to -15°C storage and thaw it at room temperature.

  NOTE
  The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- Turn on the Covaris instrument and follow the manufacturer guidelines to set up your instrument.
- Apply a CFP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a CSP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a DNA barcode label to a new 96-well 0.3 ml PCR plate.
- Apply an IMP barcode label to a new 96-well 0.3 ml PCR plate.

Make CFP

1. Illumina recommends quantifying gDNA samples using a fluorometric-based method such as Qubit or PicoGreen.
2. Illumina recommends normalizing gDNA samples with Resuspension Buffer, in a final volume of 55 µl in each well of the new 0.3 ml PCR plate labeled with the DNA barcode:
   - 20 ng/µl for a 350 bp insert size
   - 40 ng/µl for a 550 bp insert size
3. Gently pipette the entire volume up and down 10 times, then centrifuge briefly.

Fragment DNA

1. Shear the gDNA sample by transferring 52.5 µl of each DNA sample from the DNA plate to a separate, new Covaris tube:
   - 1 µg for a 350 bp insert size
   - 2 µg for a 550 bp insert size

   Use the wells of the new 0.3 ml PCR plate labeled with CFP barcode or another device to hold the Covaris tubes upright.

   NOTE
   Load the DNA sample into the Covaris tube slowly to avoid creating air bubbles. However, air bubbles might not be preventable.
2. Centrifuge the plate at 280 × g for 5 seconds.

3 Fragment the DNA using the following settings:

Table 5 Covaris S220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	5%	
Peak Incident Power	175 W	
Cycles per burst	200	
Duration	50 seconds	25 seconds
Mode	Frequency sweeping	
Temperature	5.5° to 6°C	

NOTE
The Covaris M220 settings are optimized for use with the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm.

Table 6 Covaris M220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	20%	
Peak Incident Power	50 W	
Cycles per burst	200	
Duration	65 seconds	45 seconds
Temperature	20°C	

Table 7 Covaris S2 and E210 Settings

Setting	350 bp Insert	550 bp Insert
Duty cycle	10%	
Intensity	5.0	2.0
Cycles per burst	200	
Duration	45 seconds	
Mode	Frequency sweeping	
Displayed Power	S2—23 W	S2—9 W
	E210—14 W	E210—7 W
Temperature	5.5° to 6°C	

4 Centrifuge the plate at 280 × g for 5 seconds.

5 Transfer 50 µl of fragmented DNA from each Covaris tube in the CFP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CSP barcode, using a single channel pipette.

6 Proceed immediately to *Clean Up Fragmented DNA*.

Clean Up Fragmented DNA

1 Vortex the room temperature Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 80 µl mixed Sample Purification Beads to each well. Set a 200 µl pipette to 125 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:

- If using a single channel pipette, vortex the beads after processing 4 samples
- If using a multichannel pipette, vortex the beads after processing 4 columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette

NOTE
Keep the Sample Purification Beads tube at room temperature for later use in the protocol.

3 Incubate the plate at room temperature for 5 minutes.

4 Place the plate on the magnetic stand for 8 minutes or until the liquid is clear.

5 Using a 200 µl single channel or multichannel pipette set to 125 µl, remove and discard 125 µl of the clear supernatant from each well.

NOTE
Leave the plate on the magnetic stand while performing the following steps 6–11.

6 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

7 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

8 Repeat steps 6 and 7 to perform an 80% EtOH wash 2 times.

9 Remove and discard any remaining EtOH from each well with a 10 µl pipette.

10 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

11 With the plate on the magnetic stand, add 52.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

12 Remove the plate from the magnetic stand.

13 Resuspend the beads in each well by repeatedly dispensing the Resuspension Buffer over the beads until they are immersed in the solution. Do not touch the beads with the pipette tip. Gently pipette the entire volume up and down 10 times to mix thoroughly.

14 Incubate the plate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

16 Transfer 50 µl of the clear supernatant from each well of the CSP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the IMP barcode. Take care not to disturb the beads.

NOTE
Make sure that you use a 0.3 ml PCR plate, because IMP plate volumes are greater than a 0.2 ml PCR plate. Final volumes during size selection are up to 260 µl per well.

17 Proceed immediately to *Perform End Repair and Size Selection*.

Perform End Repair and Size Selection

This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix 2. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. Following end repair, the appropriate library size is selected using different ratios of the Sample Purification Beads.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix 2 (ERP2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
[Optional] End Repair Control (CTE)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • ALP (Adapter Ligation Plate) • CEP (Clean Up End Repair Plate)	1 label per plate	15°C to 30°C	Illumina
15 ml conical tube	1	15°C to 30°C	User
96-well 0.3 ml PCR plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
PCR grade water	1 bottle	15°C to 30°C	User
RNase/DNase-free 8-tube strips and caps (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User

Preparation

- Prepare an ice bucket.

- Remove the End Repair Control from -25°C to -15°C storage and thaw it at room temperature.

  NOTE
  The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Review best practices for handling magnetic beads. See *Additional Resources* on page 6 for information about TruSeq DNA PCR-Free Library Prep best practices on the Illumina website.
- Remove the Sample Purification Beads and Resuspension Buffer from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Preprogram the thermal cycler with the following program and save as **ERP**:
  - Choose the thermal cycler preheat lid option and set to 100°C
  - 30°C for 30 minutes
  - Hold at 4°C
- Apply an ALP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a CEP barcode label to a new 96-well 0.3 ml PCR plate.

Make IMP

1. Do the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube at 600 × g for 5 seconds.
     - Add 10 µl thawed End Repair Control to each well.
   - If not using the in-line control reagent, add 10 µl Resuspension Buffer to each well.
2. Centrifuge the thawed End Repair Mix 2 tube at 600 × g for 5 seconds.
3. Add 40 µl End Repair Mix 2 to each well. Set a 200 µl pipette to 75 µl and then gently pipette the entire volume up and down 10 times to mix thoroughly.
4. Seal the IMP plate with a Microseal 'B' adhesive seal.
5. Return the End Repair Mix 2 tube to -25°C to -15°C storage.

Incubate IMP

1. Centrifuge the IMP plate at 280 × g for 1 minute.
2. Place the plate on the preprogrammed thermal cycler. Close the lid then select and run the **ERP** program. Each well contains 50 µl of sample.
   a. Choose the thermal cycler preheat lid option and set to 100°C
   b. 30°C for 30 minutes
   c. Hold at 4°C
3. Remove the IMP plate from the thermal cycler when the program reaches 4°C.

Clean Up IMP and Size Selection

1. Remove the adhesive seal from the IMP plate.

Remove Large DNA Fragments

1. Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Combine Sample Purification Beads and PCR grade water in a tube to create a diluted bead mixture of 160 µl per 100 µl of end-repaired sample:

- When processing > 6 samples at a time, use a new 15 ml conical tube
- When processing ≤ 6 samples at a time, use a new 1.7 ml microcentrifuge tube

Determine the volumes using the following formulas, which include 15% excess for multiple samples:

Table 8 Diluted Bead Mixture for a 350 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 109.25 µl	1311 µl	
PCR grade water	# of samples X 74.75 µl	897 µl	

Table 9 Diluted Bead Mixture for a 550 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 92 µl	1104 µl	
PCR grade water	# of samples X 92 µl	1104 µl	

3 Vortex the diluted bead mixture for 5 seconds to make sure that the beads are evenly dispersed.

4 Add 160 µl of the diluted bead mixture to each well of the IMP plate. Set a 200 µl pipette to 200 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Aspirate the diluted bead mixture slowly and dispense it slowly due to the viscosity of the solution. Changes in the volume of the diluted bead mixture affect the insert size of your library.

NOTE
Vortex the diluted bead mixture frequently. Illumina recommends the following:

- If using a single channel pipette, vortex the mixture after processing 4 samples
- If using a multichannel pipette, vortex the mixture after processing 4 columns
- If the mixture is in a reagent reservoir, mix with a 1000 µl pipette.

5 Incubate the plate at room temperature for 5 minutes.

6 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

7 Set a 200 µl single channel or multichannel pipette to 125 µl. **Transfer 125 µl of the supernatant, which contains the DNA of interest, from each well of the IMP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CEP barcode**. Take care not to disturb the beads.

NOTE
Transfer, do not discard, the supernatant. It contains the DNA of interest.

8 Repeat step 7. **Each CEP plate well now contains a total of 250 µl of DNA of interest.**

9 Discard the IMP plate that contains the beads.

10 Discard any remaining diluted bead mixture.

Remove Small DNA Fragments

NOTE
In the following steps, use **undiluted** Sample Purification Beads.

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 30 µl **undiluted** Sample Purification Beads to each well. Set a 200 µl pipette to 200 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Aspirate the Sample Purification Beads slowly and dispense them slowly due to the viscosity of the solution. Changes in the volume of the bead mixture affect the insert size and yield of your library.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:

- If using a single channel pipette, vortex the beads after processing 4 samples
- If using a multichannel pipette, vortex the beads after processing 4 columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette

3 Incubate the plate at room temperature for 5 minutes.

4 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

5 Using a 200 µl single channel or multichannel pipette set to 138 µl, remove and discard 138 µl of the supernatant from each well. Take care not to disturb the beads.

6 Repeat step 5 to remove and discard a total of 276 µl of the supernatant from each well.

NOTE
Leave the plate on the magnetic stand while performing the following steps 7–12.

7 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

8 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

9 Repeat steps 7 and 8 to perform an 80% EtOH wash 2 times.

10 Remove and discard any remaining EtOH with a 10 µl pipette.

11 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

12 With the plate on the magnetic stand, add 17.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

13 Remove the plate from the magnetic stand.

14 Resuspend the beads in each well by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

15 Incubate the plate at room temperature for 2 minutes.

16 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

17 Transfer 15 µl of the clear supernatant from each well of the CEP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the ALP barcode.

NOTE

Illumina recommends performing 2 consecutive transfers. This technique reduces sample loss by making sure that all of the liquid is transferred without disturbing the beads.

1. Using a 10 µl single channel or multichannel pipette set to 10 µl, transfer 10 µl of the clear supernatant from each well of the IMP plate to the corresponding well of the ALP plate.
2. Set the pipette to 5 µl and use the same pipette tip to transfer 5 µl of the clear supernatant from each well of the IMP plate to the corresponding well of the ALP plate.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3′ Ends*, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to each other during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] A-Tailing Control (CTA)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free 8-tube strips and caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature.
  - A-Tailing Control

    NOTE
    The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.

  - A-Tailing Mix
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up IMP and Size Selection*.
  - Let it thaw at room temperature.
  - Centrifuge the thawed plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the plate.

- Preprogram the thermal cycler with the following program and save as **ATAIL70**:
  - Choose the preheat lid option and set to 100°C
  - 37°C for 30 minutes
  - 70°C for 5 minutes
  - 4°C for 5 minutes
  - Hold at 4°C

Add ATL

1. Centrifuge the thawed A-Tailing Control (if using A-Tailing Control) and A-Tailing Mix tubes at 600 × g for 5 seconds.
2. Do the following:
   - If using the in-line control reagent, add 2.5 µl thawed A-Tailing Control to each well.
   - If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well.
3. Add 12.5 µl thawed A-Tailing Mix to each well. Set a 200 µl pipette to 20 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
4. Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

1. Centrifuge the ALP plate at 280 × g for 1 minute.
2. Place the plate on the preprogrammed thermal cycler. Close the lid, then select and run the **ATAIL70** program. Each well contains 30 µl of sample.
   a. Choose the preheat lid option and set to 100°C
   b. 37°C for 30 minutes
   c. 70°C for 5 minutes
   d. 4°C for 5 minutes
   e. Hold at 4°C
3. When the thermal cycler temperature has been at 4°C for 5 minutes, remove the plate from the thermal cycler.
4. Centrifuge the ALP plate at 280 × g for 1 minute.
5. Proceed immediately to *Ligate Adapters*.

Ligate Adapters

This process ligates multiple indexing adapters to the ends of the DNA fragments, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By

Choose from the following depending on the kit you are using:
• TruSeq DNA PCR-Free LT Library Prep Kit contents:
  • DNA Adapter Indexes (AD001–AD016, AD018–AD023, AD025, AD027)
• TruSeq DNA PCR-Free HT Library Prep Kit contents:
  • DAP (DNA Adapter Plate)
	1 tube of each index being used, per column of 8 reactions
	or
	1 DAP
		-25°C to -15°C	Illumina

Ligation Mix 2 (LIG2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
[Optional] Ligation Control (CTL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CAP (Clean Up ALP Plate) • DAP (DNA Adapter Plate) (if using the HT kit) • TSP1 (Target Sample Plate)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free 8-tube strips and caps (if using multichannel pipettes)	5–28	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5–28	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Appropriate DNA Adapter tubes (depending on the DNA Adapter Indexes being used) or the DAP.

    NOTE
    - Review the *TruSeq Library Prep Pooling Guide (part # 15042173)*.
    - When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
    - When indexing libraries with the DAP:
      - Review *Handling Adapter Plate* in the *TruSeq Library Prep Pooling Guide (part # 15042173)*.
      - Arrange samples that will be pooled together in the same orientation as the indexes in the DAP.
      - Illumina recommends that the RAP does not undergo more than 4 freeze-thaw cycles. To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.
  - Stop Ligation Buffer

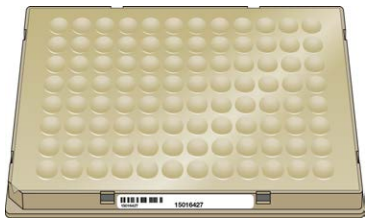
    NOTE
    Do not remove the Ligation Mix 2 tube from -25°C to -15°C storage until instructed to do so in the procedure.
  - Ligation Control

    NOTE
    The use of the Ligation Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Review best practices for handling magnetic beads. See *Additional Resources* on page 6 for information about TruSeq DNA PCR-Free Library Prep best practices on the Illumina website.
- Preprogram the thermal cycler with the following program and save as **LIG**:
  - Choose the thermal cycler preheat lid option and set to 100°C
  - 30°C for 10 minutes
  - Hold at 4°C
- Apply a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Add LIG

1. Do the following:
   - If using DNA Adapter tubes, centrifuge the thawed tubes at 600 × g for 5 seconds.
   - If using a DAP:
     - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.
     - Remove the tape seal from the plate.
     - Centrifuge the plate at 280 × g for 1 minute to collect all of the adapter to the bottom of the well.
     - Remove the plastic cover. Save the cover if you are not processing the entire plate at the same time.
     - If it is the first time using this DAP, apply the DAP barcode label to the plate.
2. Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes at 600 × g for 5 seconds.
3. Immediately before use, remove the Ligation Mix 2 tube from -25°C to -15°C storage.
4. Remove the adhesive seal from the ALP plate.
5. Do the following:
   - If using the in-line control reagent, add 2.5 µl thawed Ligation Control to each well.
   - If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well.
6. Add 2.5 µl Ligation Mix 2 to each well.
7. Return the Ligation Mix 2 tube to -25°C to -15°C storage immediately after use.
8. Do the following:
   - If using DNA Adapter tubes, add 2.5 µl thawed DNA Adapter Index to each well. Set a 200 µl pipette to 35 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
   - If using a DAP:
     - Place the DAP on the benchtop so that the part number barcode, on the long side of the plate, is facing you and the clipped corner is on the lower left.

       Figure 2 Correct DAP Orientation

     - Do the following to pierce the foil seal:
       - If using the entire plate at the same time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press the clean plate over the foil seal.
       - If using only part of the plate, use the bottom of a clean 8-tube strip, with caps attached, to pierce holes in the seals of the wells that will be used for ligation. Repeat with a new, clean 8-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.

— Using an 8-tip multichannel pipette, transfer 2.5 µl thawed DNA Adapter from the DAP well to each well of the ALP plate. Set a 200 µl pipette to 35 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.

9 Seal the plate with a Microseal 'B' adhesive seal.

10 Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 2 ALP

1 Place the sealed ALP plate on the preprogrammed thermal cycler. Close the lid then select and run the **LIG** program. Each well contains 37.5 µl of sample.
   a Choose the thermal cycler preheat lid option and set to 100°C
   b 30°C for 10 minutes
   c Hold at 4°C

2 Remove the ALP plate from the thermal cycler when the program reaches 4°C.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl Stop Ligation Buffer to each well to inactivate the ligation. Set a 200 µl pipette to 30 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 42.5 µl mixed Sample Purification Beads to each well. Set a 200 µl pipette to 60 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:
- If using a single channel pipette, vortex the beads after processing 4 samples
- If using a multichannel pipette, vortex the beads after processing 4 columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette

3 Incubate the plate at room temperature for 5 minutes.

4 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

5 Remove and discard 80 µl of the supernatant from each well. Take care not to disturb the beads.

NOTE
Leave the plate on the magnetic stand while performing the following steps 6–11.

6 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

7 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

8 Repeat steps 6 and 7 to perform an 80% EtOH wash 2 times.

9 Remove and discard any remaining EtOH from each well with a 10 µl pipette.

10 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

11 With the plate on the magnetic stand, add 52.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

12 Remove the plate from the magnetic stand.

13 Resuspend the beads in each well by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

14 Incubate the plate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

16 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode. Take care not to disturb the beads.

17 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

18 Add 50 µl mixed Sample Purification Beads to each well for a second cleanup. Set a 200 µl pipette to 75 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

19 Incubate the plate at room temperature for 5 minutes.

20 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

21 Remove and discard 95 µl of the supernatant from each well. Take care not to disturb the beads.

NOTE
Leave the plate on the magnetic stand while performing the following steps 22–27.

22 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

23 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

24 Repeat steps 22 and 23 to perform an 80% EtOH wash 2 times.

25 Remove and discard any remaining EtOH from each well with a 10 µl pipette.

26 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

27 With the plate on the magnetic stand, add 22.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

28 Remove the plate from the magnetic stand.

29 Resuspend the beads in each well by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

30 Incubate the plate at room temperature for 2 minutes.

31 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

32 Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the TSP1 barcode. Take care not to disturb the beads.

33 Return the following to -25°C to -15°C storage:

- Ligation Control
- DNA Adapter Indexes
- DAP
- Stop Ligation Buffer

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Libraries*, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Validate Libraries

Illumina recommends performing the following procedures for quality control analysis on the sample libraries and quantification of the DNA libraries.

Quantify Libraries

To achieve the highest quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantitation of DNA libraries. qPCR must be used to quantify TruSeq DNA PCR-Free Library Prep libraries.

NOTE
Methods other than qPCR quantify molecules that do not have adapters on both ends and do not form clusters. More of these nonclusterable molecules can be present due to the absence of PCR enrichment and quantification by methods other than qPCR can be inaccurate.

NOTE
TruSeq DNA PCR-Free Library Prep library quantitation has been validated using the KAPA Library Quantification Kit specified in the *Consumables and Equipment* on page 72.

Follow qPCR instructions included in the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* using the KAPA standard (www.kapabiosystems.com), with the following modifications:

NOTE
You can download the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* from the Kapa Biosystems website (www.kapabiosystems.com).

- Use at least 2 µl of the original library stock in the library dilution step to ensure accurate and reproducible quantitation.
- Illumina recommends 2 further independent (not serial) 1:10,000 and 1:20,000 dilutions using at least 2 µl of the initial diluted libraries to evaluate quantitation precision.

NOTE
For guidance on handling small liquid volumes, see *Handling Liquids* in the TruSeq DNA PCR-Free Library Prep Best Practices. See *Additional Resources* on page 6 for information TruSeq DNA PCR-Free Library Prep Best Practices on the Illumina website.

The concentration of each library is calculated as indicated in Table 10 and Table 11:

- Obtain the calculated concentration of the 1:10,000 and 1:20,000 dilutions of the library, as determined by qPCR, in relation to the concentrations of the correctly annotated KAPA DNA Standards 1–6. Use the average of the replicate data points to determine the concentration of the diluted library.
- Perform a size adjustment calculation to account for the difference in size between the average fragment length of the library and the KAPA DNA Standard (452 bp):
  - For 350 bp libraries, use 470 bp for the average fragment length
  - For 550 bp libraries, use 670 bp for the average fragment length

NOTE
Do not use the average fragment length of the library insert size based on the Bioanalyzer results. PCR-free library fragment sizes measured on the Bioanalyzer are substantially larger than would be predicted or derived from sequencing data.

- Calculate the concentration of the undiluted library by taking account of the relevant dilution factor (e.g. 1:10,000 and 1:20,000). Use the average of the replicate data points

corresponding to each library DNA dilution to calculate the concentration of the undiluted library.

- If 1 of the replicates appears to be an outlier, it can be omitted from the calculation. If more than 1 of replicates appears to be outliers, repeat the assay.

Table 10 350 bp Library Concentration Calculation

Dilution Factor	Calculated by qPCR instrument (pM)*		Average diluted library (pM)	Size adjusted diluted library (pM)	Undiluted library (pM)*	Undiluted library (pM)
1:10,000	A1	A2	A = (A1 + A2)/2	W1 = A x (452/470)	C1 = W1 x 10,000	(C1 + C2)/2
1:20,000	B1	B2	B = (B1 + B2)/2	W2 = B x (452/470)	C2 = W2 x 20,000	

Table 11 550 bp Library Concentration Calculation

Dilution Factor	Calculated by qPCR instrument (pM)*		Average diluted library (pM)	Size adjusted diluted library (pM)	Undiluted library (pM)*	Undiluted library (pM)
1:10,000	C1	C2	C = (C1 + C2)/2	W3 = C x (452/670)	C3 = W3 x 10,000	(C3 + C4)/2
1:20,000	D1	D2	D = (D1 + D2)/2	W4 = D x (452/670)	C4 = W4 x 20,000	

*Duplicate data points

Quality Control

To verify the size of your fragments, check the template size distribution.

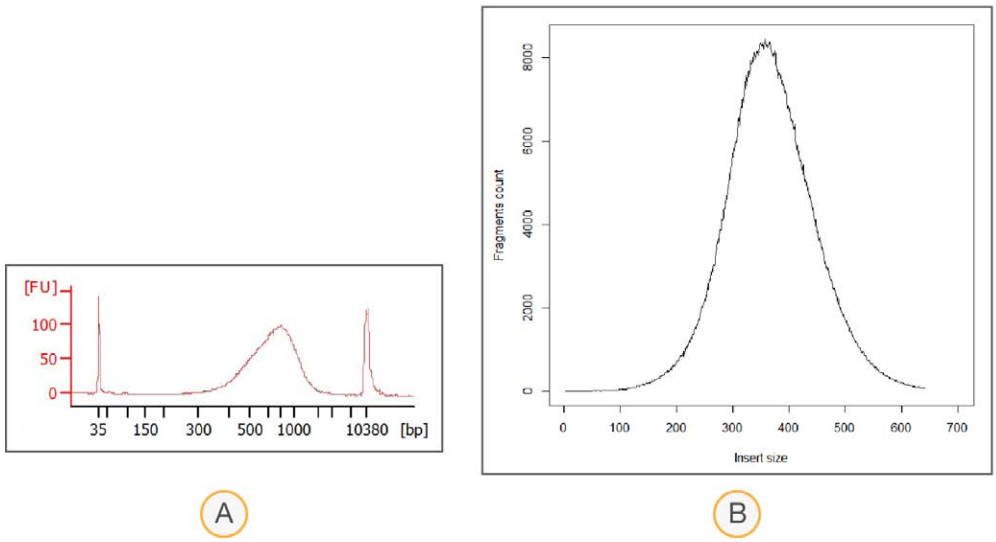
1 Prepare a 1:5 dilution of the DNA library with water.

2 Run 1 µl of the diluted DNA library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.

Run samples on a Bioanalyzer for qualitative purposes only.

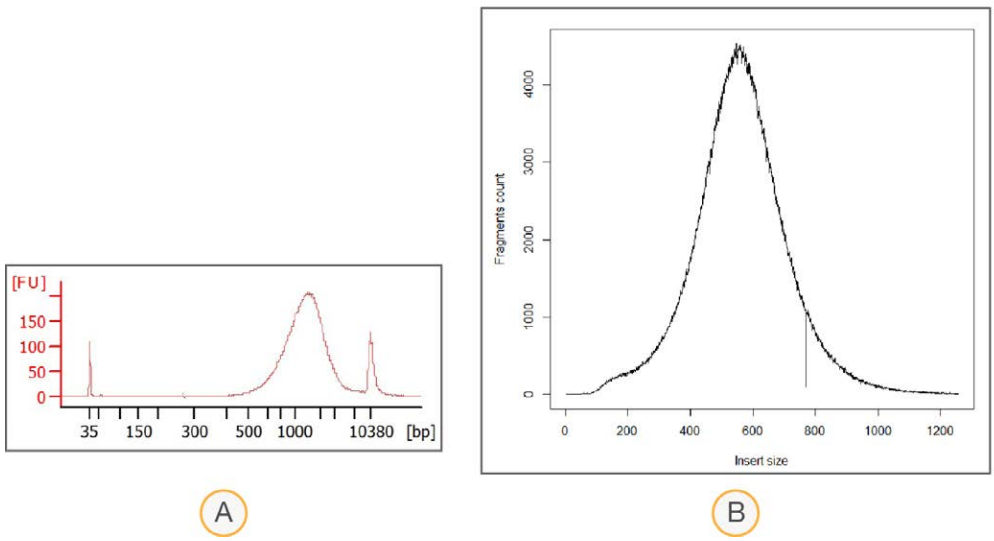
When performing quality control on TruSeq DNA PCR-Free Library Prep libraries, PCR-Free library fragment sizes measured on the Bioanalyzer are substantially larger than would be predicted or derived from sequencing data. The larger size is due to anomalous migration of fragments on the chip due to the presence of certain structural features, which would normally be removed if a subsequent PCR-enrichment step were performed. Figure 3 and Figure 4 show a comparison between library fragment sizes derived by a Bioanalyzer and the corresponding insert sizes derived from the alignment of paired-end reads to a suitable reference sequence.

Figure 3 Example TruSeq DNA PCR-Free Library Prep 350 bp Insert Library Distribution



A Bioanalyzer
B Paired-End Alignment

Figure 4 Example TruSeq DNA PCR-Free Library Prep 550 bp Insert Library Distribution



A Bioanalyzer
B Paired-End Alignment

Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 2 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-indexed DNA libraries are normalized to 2 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only)	1 label per plate	15°C to 30°C	Illumina
96-well midi plate (for pooling only, if pooling > 40 samples)	1	15°C to 30°C	User
96-well 0.3 ml PCR plates	2 (second plate for pooling only, if pooling ≤ 40 samples)	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each library to 2 nM	15°C to 30°C	User

Preparation

- Remove the TSP1 plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up ALP*.
  - Let it thaw at room temperature.
  - Centrifuge the thawed plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed plate.
- Apply a DCT barcode label to a new 96-well 0.3 ml PCR plate.
- [For pooling only] Review the *TruSeq Library Prep Pooling Guide (part # 15042173)*.
- [For pooling only] Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate if pooling ≤ 40 samples or a 96-well midi plate if pooling > 40 samples.

Make DCT

1 Transfer 5 µl of the library from each well of the TSP1 plate to the corresponding well of the new 0.3 ml PCR plate labeled with the DCT barcode.

2 Normalize the library concentration in each well to 2 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

NOTE
Depending on the yield quantification data of each library, the final volume in the plate can vary from 5–100 µl.

3 Gently pipette the entire volume up and down 10 times to mix thoroughly.

4 Do the following, depending on the type of library you want to generate:
- For libraries that are not pooled, the protocol stops here. Do the following:
  - To continue immediately to sequencing, proceed to cluster generation. For more information, see the cluster generation section of the system guide for your Illumina platform.
  - To stop here before sequencing, seal the DCT plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.
- For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)

NOTE
Do not make a PDP plate if you are not pooling samples.

1 Determine the number of samples to be combined together for each pool.

NOTE
Avoid pooling 2 samples with the same index.

2 Do the following:
- If pooling 2–24 samples:
  - Transfer 5 µl of each normalized library to be pooled from the DCT plate to 1 well of the new 0.3 ml PCR plate labeled with the PDP barcode.

  The total volume in each well is 5 X the number of combined sample libraries and 10–120 µl (2–24 libraries). For example, the volume for 2 samples is 10 µl, the volume for 12 samples is 60 µl, or the volume for 24 samples is 120 µl.
- If pooling 25–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized library in column 1 of the DCT plate to column 1 of the new 0.3 ml PCR or midi plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized library in column 2 of the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Gently pipette the entire volume of each well of column 1 up and down 10 times to mix thoroughly.
  - Combine the contents of each well of column 1 into well A2 of the PDP plate for the final pool.

3 Gently pipette the entire volume up and down 10 times to mix thoroughly.

4 Do the following:
- To continue immediately to sequencing, proceed to cluster generation. For more information, see the system guide for your Illumina sequencing platform.
- To stop here before sequencing, seal the PDP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.

High Sample (HS) Protocol

Chapter 3

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Introduction

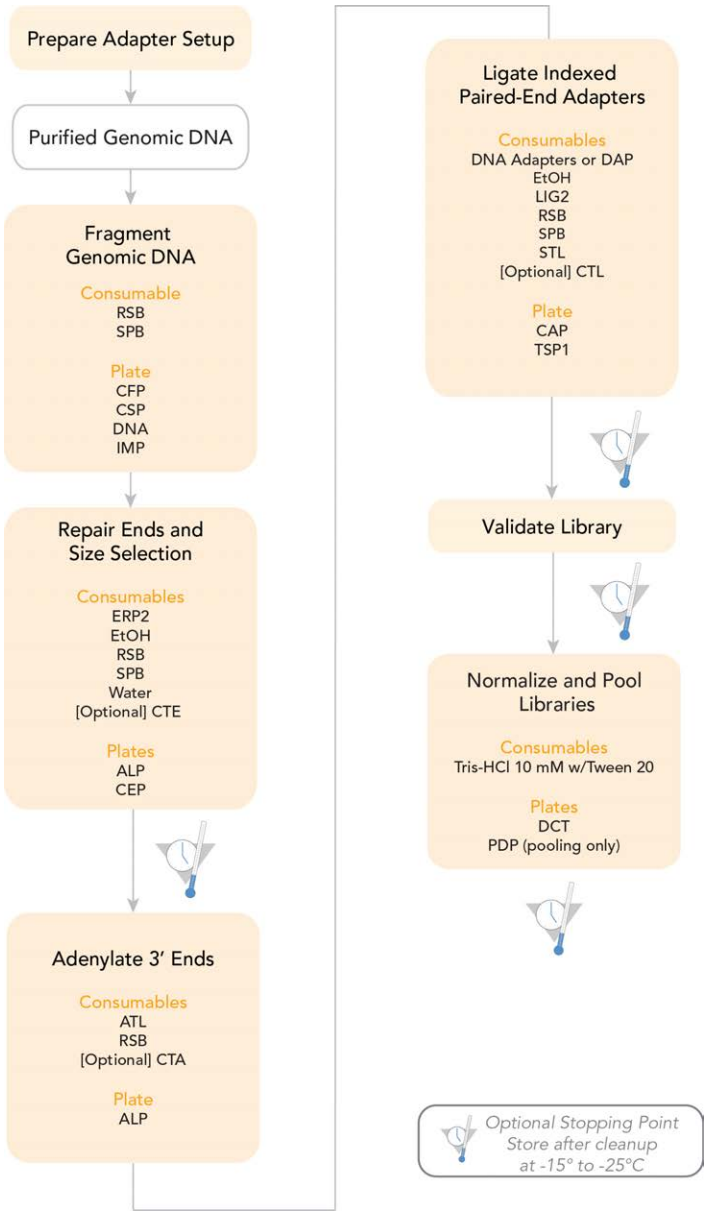
This chapter describes the TruSeq DNA PCR-Free Library Prep HS protocol. This protocol is intended for preparing more than 24 samples at the same time using either the LT or HT kit.

- Follow the protocols in the order described, using the specified volumes and incubation parameters.
- Review Best Practices before proceeding. See *Additional Resources* on page 6 for information on how to access TruSeq DNA PCR-Free Library Prep Best Practices on the Illumina website.
- Review Appendix A Supporting Information before proceeding, to confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the HS protocol.
- This HS protocol requires shaking and heating equipment to mix reagents and for incubation (see *User-Supplied Equipment - Additional Items for HS Processing* on page 74).

Library Prep Workflow

The following figure illustrates theTruSeq DNA PCR-Free Library Prep HS workflow.

Figure 5 TruSeq DNA PCR-Free Library Prep HS Workflow



Prepare Adapter Setup

If you are pooling, use IEM or BaseSpace to record information about your samples before beginning library prep.

- Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software.
- Use BaseSpace to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software.

NOTE

For information about IEM and BaseSpace software documentation on the Illumina website, see *Additional Resources* on page 6.

Review the planning steps in the *TruSeq Library Prep Pooling Guide (part # 15042173)* when preparing libraries for Illumina sequencing systems that require balanced index combinations.

Fragment DNA

This process describes how to optimally fragment the gDNA depending on the Covaris platform used. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process is optimized to obtain final libraries with the following average insert sizes:

Table 12 Insert Size Options

Insert Size	350 bp	550 bp
Input DNA Per Sample	1 µg	2 µg
Recommended Read Length	≤ 2 x 101 bp	≤ 2 x 151 bp*

* Read lengths greater than 2 x 151 bp produce a significantly higher percentage of overlapping read-pairs.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C (2°C to 8°C after initial thaw)	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Barcode labels for: • CFP (Covaris Fragmentation Plate) • CSP (Clean Up Sheared DNA Plate) • DNA (DNA Plate) • IMP (Insert Modification Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well midi plates	3	15°C to 30°C	User
Covaris tubes	1 per sample	15°C to 30°C	User
DNA samples	• 1 µg per sample for a 350 bp insert size • 2 µg per sample for a 550 bp insert size	-25°C to -15°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Preparation

- Review *DNA Input Recommendations* on page 4.
- Review best practices for handling magnetic beads. See *Additional Resources* on page 6 for information about TruSeq DNA PCR-Free Library Prep best practices on the Illumina website.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the Resuspension Buffer from -25°C to -15°C storage and thaw it at room temperature.

  NOTE
  The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- Turn on the Covaris instrument and follow the manufacturer guidelines to set up your instrument.
- Calibrate the microplate shaker with a stroboscope and set it to 1800 rpm.
- Apply a CFP barcode label to a new 96-well HSP plate
- Apply a CSP barcode label to a new 96-well midi plate.
- Apply a DNA barcode label to a new 96-well midi plate.
- Apply an IMP barcode label to a new 96-well midi plate.

Make CFP

1. Illumina recommends quantifying gDNA samples using a fluorometric-based method such as Qubit or PicoGreen.
2. Illumina recommends normalizing gDNA samples with Resuspension Buffer, to a final volume of 55 µl in each well of the new midi plate labeled with the DNA barcode:
   - 20 ng/µl for a 350 bp insert size
   - 40 ng/µl for a 550 bp insert size
3. Mix thoroughly as follows:
   a. Seal the plate with a Microseal 'B' adhesive seal.
   b. Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.
4. Centrifuge the DNA plate at 280 × g for 1 minute.

Fragment DNA

1. Remove the adhesive seal from the DNA plate.
2. Shear the gDNA sample by transferring 52.5 µl of each DNA sample from the DNA plate to a separate, new Covaris tube:
   - 1 µg for a 350 bp insert size
   - 2 µg for a 550 bp insert size

   Use the wells of the new HSP plate labeled with CFP barcode or another device to hold the Covaris tubes upright.

   NOTE
   Load the DNA sample into the Covaris tube slowly to avoid creating air bubbles. However, air bubbles might not be preventable.
3. Centrifuge the CFP plate at 280 × g for 5 seconds.

4 Fragment the DNA using the following settings:

Table 13 Covaris S220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	5%	
Peak Incident Power	175 W	
Cycles per burst	200	
Duration	50 seconds	25 seconds
Mode	Frequency sweeping	
Temperature	5.5° to 6°C	

NOTE
The Covaris M220 settings are optimized for use with the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm.

Table 14 Covaris M220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	20%	
Peak Incident Power	50 W	
Cycles per burst	200	
Duration	65 seconds	45 seconds
Temperature	20°C	

Table 15 Covaris S2 and E210 Settings

Setting	350 bp Insert	550 bp Insert
Duty cycle	10%	
Intensity	5.0	2.0
Cycles per burst	200	
Duration	45 seconds	
Mode	Frequency sweeping	
Displayed Power	S2—23 W	S2—9 W
	E210—14 W	E210—7 W
Temperature	5.5° to 6°C	

5 Centrifuge the plate at 280 × g for 5 seconds.

6 Transfer 50 µl of fragmented DNA from each Covaris tube in the CFP plate to the corresponding well of the new midi plate labeled with the CSP barcode, using a single channel pipette.

7 Proceed immediately to *Clean Up Fragmented DNA*.

Clean Up Fragmented DNA

1 Vortex the room temperature Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 80 µl mixed Sample Purification Beads to each well. Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:
- If using a single channel pipette, vortex the beads after processing 4 samples
- If using a multichannel pipette, vortex the beads after processing 4 columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette

NOTE
Keep the Sample Purification Beads tube at room temperature for later use in the protocol.

3 Incubate the plate at room temperature for 5 minutes.

4 Centrifuge the plate at 280 × g for 1 minute.

5 Remove the adhesive seal from the plate.

6 Place the plate on the magnetic stand for 8 minutes or until the liquid is clear.

7 Using a 200 µl single channel or multichannel pipette set to 125 µl, remove and discard 125 µl of the clear supernatant from each well. Take care not to disturb the beads.

NOTE
Leave the plate on the magnetic stand while performing the following steps 8–13.

8 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

9 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

10 Repeat steps 8 and 9 to perform an 80% EtOH wash 2 times.

11 Remove and discard any remaining EtOH from each well with a 10 µl pipette.

12 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

13 With the plate on the magnetic stand, add 52.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

14 Remove the plate from the magnetic stand.

15 Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

16 Incubate the plate at room temperature for 2 minutes.

17 Centrifuge the plate at 280 × g for 1 minute.

18 Remove the adhesive seal from the plate.

19 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

20 Transfer 50 µl of the clear supernatant from each well of the CSP plate to the corresponding well of the new midi plate labeled with the IMP barcode. Take care not to disturb the beads.

21 Proceed immediately to *Perform End Repair and Size Selection*.

Perform End Repair and Size Selection

This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix 2. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. Following end repair, the appropriate library size is selected using different ratios of the Sample Purification Beads.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix 2 (ERP2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
[Optional] End Repair Control (CTE)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • ALP (Adapter Ligation Plate) • CEP (Clean Up End Repair Plate)	1 label per plate	15°C to 30°C	Illumina
15 ml conical tube	1	15°C to 30°C	User
96-well midi plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	5	15°C to 30°C	User
PCR grade water	1 bottle	15°C to 30°C	User
RNase/DNase-free 8-tube strips and caps (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the End Repair Mix 2 from -25°C to -15°C storage. Thaw it at room temperature and then place it on ice.
- Remove the End Repair Control from -25°C to -15°C storage and thaw it at room

temperature.

NOTE
The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Remove the Sample Purification Beads and Resuspension Buffer from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Review best practices for handling magnetic beads. See *Additional Resources* on page 6 for information about TruSeq DNA PCR-Free Library Prep best practices on the Illumina website.
- Preheat the microheating system to 30°C.
- Apply an ALP barcode label to a new 96-well midi plate.
- Apply a CEP barcode label to a new 96-well midi plate.

Make IMP

1. Do the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube at 600 × g for 5 seconds.
     - Add 10 µl thawed End Repair Control to each well.
   - If not using the in-line control reagent, add 10 µl Resuspension Buffer to each well.
2. Centrifuge the thawed End Repair Mix 2 tube at 600 × g for 5 seconds.
3. Add 40 µl End Repair Mix to each well. Mix thoroughly as follows:
   a. Seal the plate with a Microseal 'B' adhesive seal.
   b. Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.
4. Centrifuge the IMP plate at 280 × g for 1 minute.
5. Return the End Repair Mix 2 tube to -25°C to -15°C storage.

Incubate IMP

1. Place the IMP plate on the preheated microheating system. Close the lid and incubate at 30°C for 30 minutes. Each well contains 50 µl of sample.
2. Remove the IMP plate from the microheating system and place the plate on ice until you are ready for the next step.

Clean Up IMP and Size Selection

1. Remove the adhesive seal from the IMP plate.

Remove Large DNA Fragments

1. Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Combine Sample Purification Beads and PCR grade water in a tube to create a diluted bead mixture of 160 µl per 100 µl of end-repaired sample:

- When processing > 6 samples at a time, use a new 15 ml conical tube
- When processing ≤ 6 samples at a time, use a new 1.7 ml microcentrifuge tube

Determine the volumes using the following formulas, which include 15% excess for multiple samples:

Table 16 Diluted Bead Mixture for a 350 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 109.25 µl	1311 µl	
PCR grade water	# of samples X 74.75 µl	897 µl	

Table 17 Diluted Bead Mixture for a 550 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 92 µl	1104 µl	
PCR grade water	# of samples X 92 µl	1104 µl	

3 Vortex the diluted bead mixture for 5 seconds to make sure that the beads are evenly dispersed.

4 Add 160 µl of the diluted bead mixture to each well of the IMP plate. Mix thoroughly as follows:

a Seal the plate with a Microseal 'B' adhesive seal.

b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE

Aspirate the diluted bead mixture slowly and dispense it slowly due to the viscosity of the solution. Changes in the volume of the diluted bead mixture affect the insert size of your library.

NOTE

Vortex the diluted bead mixture frequently. Illumina recommends the following:

- If using a single channel pipette, vortex the mixture after processing 4 samples
- If using a multichannel pipette, vortex the mixture after processing 4 columns
- If the mixture is in a reagent reservoir, mix with a 1000 µl pipette.

5 Incubate the plate at room temperature for 5 minutes.

6 Centrifuge the plate at 280 × g for 1 minute.

7 Remove the adhesive seal from the plate.

8 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

9 Set a 200 µl single channel or multichannel pipette to 125 µl. **Transfer 125 µl of the supernatant, which contains the DNA of interest, from each well of the IMP plate to the corresponding well of the new midi plate labeled with the CEP barcode**. Take care not to disturb the beads.

NOTE
Transfer, do not discard, the supernatant. It contains the DNA of interest.

10 Repeat step 9. **Each CEP plate well now contains a total of 250 µl of DNA of interest.**

11 Discard the IMP plate that contains the beads.

12 Discard any remaining diluted bead mixture.

Remove Small DNA Fragments

NOTE
In the following steps, use **undiluted** Sample Purification Beads.

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 30 µl **undiluted** Sample Purification Beads to each well of the CEP plate. Mix thoroughly as follows:

a Seal the plate with a Microseal 'B' adhesive seal.

b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE
Aspirate the Sample Purification Beads slowly and dispense them slowly due to the viscosity of the solution. Changes in the volume of the bead mixture affect the insert size of your library.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. If the beads are in a reagent reservoir, Illumina recommends mixing with a 1000 µl pipette.

3 Incubate the plate at room temperature for 5 minutes.

4 Centrifuge the plate at 280 × g for 1 minute.

5 Remove the adhesive seal from the plate.

6 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

7 Using a 200 µl single channel or multichannel pipette set to 138 µl, remove and discard 138 µl of the supernatant from each well. Take care not to disturb the beads.

8 Repeat step 7 to remove and discard a total of 276 µl of supernatant from each well.

NOTE
Leave the plate on the magnetic stand while performing the following steps 9–14.

9 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

10 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

11 Repeat steps 9 and 10 to perform an 80% EtOH wash 2 times.

12 Remove and discard any remaining EtOH with a 10 µl pipette.

13 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

14 With the plate on the magnetic stand, add 17.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

15 Remove the plate from the magnetic stand.

16 Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

17 Incubate the plate at room temperature for 2 minutes.

18 Centrifuge the plate at 280 × g for 1 minute.

19 Remove the adhesive seal from the plate.

20 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

21 Transfer 15 µl of the clear supernatant from each well of the CEP plate to the corresponding well of the new midi plate labeled with the ALP barcode.

NOTE

Illumina recommends performing 2 consecutive transfers. This technique reduces sample loss by making sure that all of the liquid is transferred without disturbing the beads.

1. Using a 10 µl single channel or multichannel pipette set to 10 µl, transfer 10 µl of the clear supernatant from each well of the IMP plate to the corresponding well of the ALP plate.
2. Set the pipette to 5 µl and use the same pipette tip to transfer 5 µl of the clear supernatant from each well of the IMP plate to the corresponding well of the ALP plate.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* immediately, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to each other during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] A-Tailing Control (CTA)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free 8-tube strips and caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature.
  - A-Tailing Control

    NOTE
    The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.

  - A-Tailing Mix
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up IMP and Size Selection*.
  - Let it thaw at room temperature.
  - Centrifuge the thawed plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the plate.
- Preheat 2 microheating systems: system 1 to 37°C and system 2 to 70°C.

Add ATL

1 Centrifuge the thawed A-Tailing Control (if using A-Tailing Control) and A-Tailing Mix tubes at 600 × g for 5 seconds.

2 Do the following:
   - If using the in-line control reagent, add 2.5 µl thawed A-Tailing Control to each well.
   - If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well.

3 Add 12.5 µl thawed A-Tailing Mix to each well. Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 1 ALP

1 Place the sealed ALP plate on the preheated microheating system 1. Close the lid and incubate at 37°C for 30 minutes. Each well contains 30 µl of sample.

2 Immediately after the 37°C incubation, remove the plate from system 1 and place the plate on the preheated microheating system 2. Close the lid and incubate at 70°C for 5 minutes.

3 Set the microheating system 1 to 30°C in preparation for *Ligate Adapters*.

4 Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 5 minutes.

5 Proceed immediately to *Ligate Adapters*.

Ligate Adapters

This process ligates indexing adapters to the ends of the DNA fragments, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By

Choose from the following depending on the kit you are using:
• TruSeq DNA PCR-Free LT Library Prep Kit contents:
  • DNA Adapter Indexes (AD001–AD016, AD018–AD023, AD025, AD027)
• TruSeq DNA PCR-Free HT Library Prep Kit contents:
  • DAP (DNA Adapter Plate)
	1 tube of each index being used, per column of 8 reactions
	or
	1 DAP
		-25°C to -15°C	Illumina

Ligation Mix 2 (LIG2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-25°C to -15°C	Illumina
[Optional] Ligation Control (CTL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CAP (Clean Up ALP Plate) • DAP (DNA Adapter Plate) (if using the HT kit) • TSP1 (Target Sample Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well midi plate	1	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free 8-tube strips and caps (if using multichannel pipettes)	5–28	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5–28	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Appropriate DNA Adapter tubes (depending on the DNA Adapter Indexes being used) or the DAP.

    NOTE
    - Review the *TruSeq Library Prep Pooling Guide (part # 15042173)*.
    - When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
    - When indexing libraries with the DAP:
      - Review *Handling Adapter Plate* in the *TruSeq Library Prep Pooling Guide (part # 15042173)*.
      - Arrange samples that will be pooled together in the same orientation as the indexes in the DAP.
      - Illumina recommends that the RAP does not undergo more than 4 freeze-thaw cycles. To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.
  - Stop Ligation Buffer

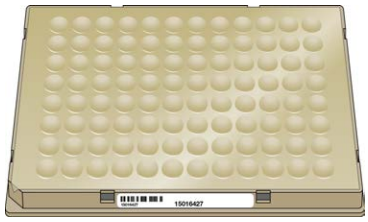
    NOTE
    Do not remove the Ligation Mix 2 tube from -25°C to -15°C storage until instructed to do so in the procedure.
  - Ligation Control

    NOTE
    The use of the Ligation Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Review best practices for handling magnetic beads. See *Additional Resources* on page 6 for information about TruSeq DNA PCR-Free Library Prep best practices on the Illumina website.
- Preheat the microheating system 1 to 30°C.
- Apply a CAP barcode label to a new 96-well midi plate.
- Apply a TSP1 barcode label to a new 96-well HSP plate.

Add LIG

1. Do the following:
   - If using DNA Adapter tubes, centrifuge the thawed tubes at 600 × g for 5 seconds.
   - If using a DAP:
     - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.
     - Remove the tape seal from the plate.
     - Centrifuge the plate at 280 × g for 1 minute to collect all of the adapter to the bottom of the well.
     - Remove the plastic cover. Save the cover if you are not processing the entire plate at the same time.
     - If it is the first time using this DAP, apply the DAP barcode label to the plate.
2. Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes at 600 × g for 5 seconds.
3. Immediately before use, remove the Ligation Mix 2 tube from -25°C to -15°C storage.
4. Remove the adhesive seal from the ALP plate.
5. Do the following:
   - If using the in-line control reagent, add 2.5 µl thawed Ligation Control to each well.
   - If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well.
6. Add 2.5 µl Ligation Mix 2 to each well.
7. Return the Ligation Mix 2 tube to -25°C to -15°C storage immediately after use.
8. Do the following:
   - If using DNA Adapter tubes, add 2.5 µl thawed DNA Adapter Index to each well.
   - If using a DAP:
     - Place the DAP on the benchtop so that the part number barcode, on the long side of the plate, is facing you and the clipped corner is on the lower left.

       Figure 6 Correct DAP Orientation

     - Do the following to pierce the foil seal:
       - If using the entire plate at the same time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press the clean plate over the foil seal.
       - If using only part of the plate, use the bottom of a clean 8-tube strip, with caps attached, to pierce holes in the seals of the wells that will be used for ligation. Repeat with a new, clean 8-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.
     - Using an 8-tip multichannel pipette, transfer 2.5 µl thawed DNA Adapter from the DAP well to each well of the ALP plate.

9 Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

10 Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 2 ALP

1 Place the sealed ALP plate on the preheated microheating system. Close the lid and incubate at 30°C for 10 minutes. Each well contains 37.5 µl of sample.

2 Remove the ALP plate from the microheating system and place the plate on ice until you are ready for the next step.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl Stop Ligation Buffer to each well to inactivate the ligation mix. Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

3 Centrifuge the ALP plate at 280 × g for 1 minute.

Clean Up ALP

1 Remove the adhesive seal from the ALP plate.

2 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

3 Add 42.5 µl mixed Sample Purification Beads to each well. Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:
- If using a single channel pipette, vortex the beads after processing 4 samples
- If using a multichannel pipette, vortex the beads after processing 4 columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette

4 Incubate the plate at room temperature for 5 minutes.

5 Centrifuge the plate at 280 × g for 1 minute.

6 Remove the adhesive seal from the plate.

7 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

8 Remove and discard 80 µl of the supernatant from each well. Take care not to disturb the beads.

NOTE
Leave the plate on the magnetic stand while performing the following steps 9–14.

9 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Do not disturb the beads.

10 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

11 Repeat steps 9 and 10 to perform an 80% EtOH wash 2 times.

12 Remove and discard any remaining EtOH from each well with a 10 µl pipette.

13 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

14 With the plate on the magnetic stand, add 52.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

15 Remove the plate from the magnetic stand.

16 Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

17 Incubate the plate at room temperature for 2 minutes.

18 Centrifuge the plate at 280 × g for 1 minute.

19 Remove the adhesive seal from the plate.

20 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

21 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new midi plate labeled with the CAP barcode. Take care not to disturb the beads.

22 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

23 Add 50 µl mixed Sample Purification Beads to each well. Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

24 Incubate the plate at room temperature for 5 minutes.

25 Centrifuge the plate at 280 × g for 1 minute.

26 Remove the adhesive seal from the plate.

27 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

28 Remove and discard 95 µl of the supernatant from each well. Take care not to disturb the beads.

NOTE
Leave the plate on the magnetic stand while performing the following steps 29–34.

29 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Take care not to disturb the beads.

30 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

31 Repeat steps 29 and 30 to perform an 80% EtOH wash 2 times.

32 Remove and discard any remaining EtOH from each well with a 10 µl pipette.

33 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Do not allow the beads to crack.

34 With the plate on the magnetic stand, add 22.5 µl Resuspension Buffer to each well. Make sure the Resuspension Buffer runs over the beads. Do not touch the beads with the pipette tip.

35 Remove the plate from the magnetic stand.

36 Mix thoroughly as follows:
   a Seal the plate with a Microseal 'B' adhesive seal.
   b Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.

37 Incubate the plate at room temperature for 2 minutes.

38 Centrifuge the plate at 280 × g for 1 minute.

39 Remove the adhesive seal from the plate.

40 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

41 Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled with the TSP1 barcode. Take care not to disturb the beads.

42 Return the following to -25°C to -15°C storage:
- Ligation Control
- DNA Adapter Indexes
- DAP
- Stop Ligation Buffer

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library*, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on the sample libraries and quantification of the DNA library templates.

Quantify Libraries

To achieve the highest quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantitation of DNA libraries. qPCR must be used to quantify TruSeq DNA PCR-Free Library Prep libraries.

NOTE
Methods other than qPCR quantify molecules that do not have adapters on both ends and do not form clusters. More of these nonclusterable molecules can be present due to the absence of PCR enrichment and quantification by methods other than qPCR can be inaccurate.

NOTE
TruSeq DNA PCR-Free Library Prep library quantitation has been validated using the KAPA Library Quantification Kit specified in the *Consumables and Equipment* on page 72.

Follow qPCR instructions included in the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* using the KAPA standard (www.kapabiosystems.com), with the following modifications:

NOTE
You can download the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* from the Kapa Biosystems website (www.kapabiosystems.com).

- Use at least 2 µl of the original library stock in the library dilution step to ensure accurate and reproducible quantitation.
- Illumina recommends 2 further independent (not serial) 1:10,000 and 1:20,000 dilutions using at least 2 µl of the initial diluted libraries to evaluate quantitation precision.

  NOTE
  For guidance on handling small liquid volumes, see *Handling Liquids* in the TruSeq DNA PCR-Free Library Prep Best Practices. See *Additional Resources* on page 6 for information TruSeq DNA PCR-Free Library Prep Best Practices on the Illumina website.

The concentration of each library is calculated as indicated in Table 18 and Table 19:

- Obtain the calculated concentration of the 1:10,000 and 1:20,000 dilutions of the library, as determined by qPCR, in relation to the concentrations of the correctly annotated KAPA DNA Standards 1–6. Use the average of the replicate data points to determine the concentration of the diluted library.
- Perform a size adjustment calculation to account for the difference in size between the average fragment length of the library and the KAPA DNA Standard (452 bp):
  - For 350 bp libraries, use 470 bp for the average fragment length
  - For 550 bp libraries, use 670 bp for the average fragment length

    NOTE
    Do not use the average fragment length of the library insert size based on the Bioanalyzer results. PCR-free library fragment sizes measured on the Bioanalyzer are substantially larger than would be predicted or derived from sequencing data.
- Calculate the concentration of the undiluted library by taking account of the relevant dilution factor (e.g. 1:10,000 and 1:20,000). Use the average of the replicate data points

corresponding to each library DNA dilution to calculate the concentration of the undiluted library.

- If 1 of the replicates appears to be an outlier, it can be omitted from the calculation. If more than 1 of replicates appears to be outliers, repeat the assay.

Table 18 350 bp Library Concentration Calculation

Dilution Factor	Calculated by qPCR instrument (pM)*		Average diluted library (pM)	Size adjusted diluted library (pM)	Undiluted library (pM)*	Undiluted library (pM)
1:10,000	A1	A2	A = (A1 + A2)/2	W1 = A x (452/470)	C1 = W1 x 10,000	(C1 + C2)/2
1:20,000	B1	B2	B = (B1 + B2)/2	W2 = B x (452/470)	C2 = W2 x 20,000	

Table 19 550 bp Library Concentration Calculation

Dilution Factor	Calculated by qPCR instrument (pM)*		Average diluted library (pM)	Size adjusted diluted library (pM)	Undiluted library (pM)*	Undiluted library (pM)
1:10,000	C1	C2	C = (C1 + C2)/2	W3 = C x (452/670)	C3 = W3 x 10,000	(C3 + C4)/2
1:20,000	D1	D2	D = (D1 + D2)/2	W4 = D x (452/670)	C4 = W4 x 20,000	

*Duplicate data points

Quality Control

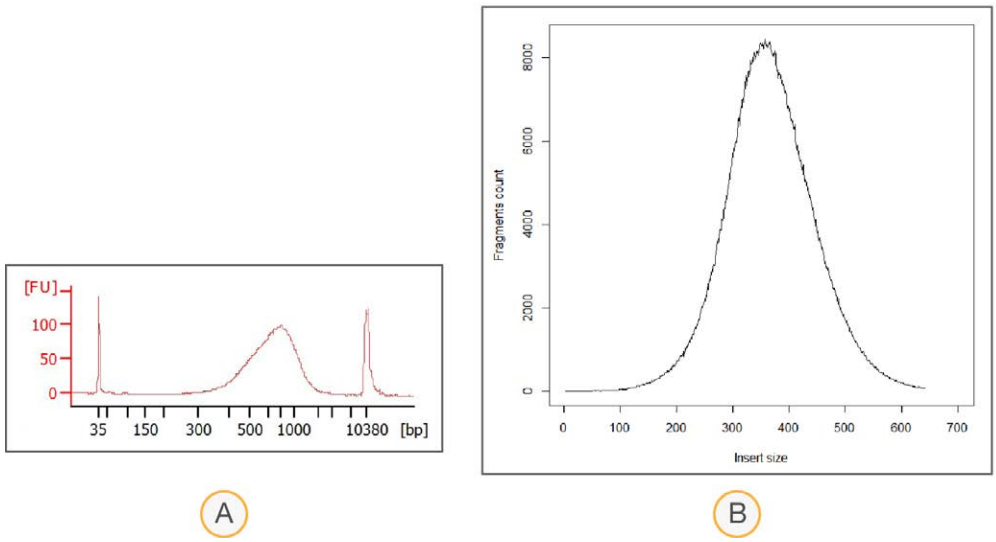
To verify the size of your fragments, check the template size distribution.

1 Prepare a 1:5 dilution of the DNA library with water.

2 Run 1 µl of the diluted DNA library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.
Run samples on a Bioanalyzer for qualitative purposes only.

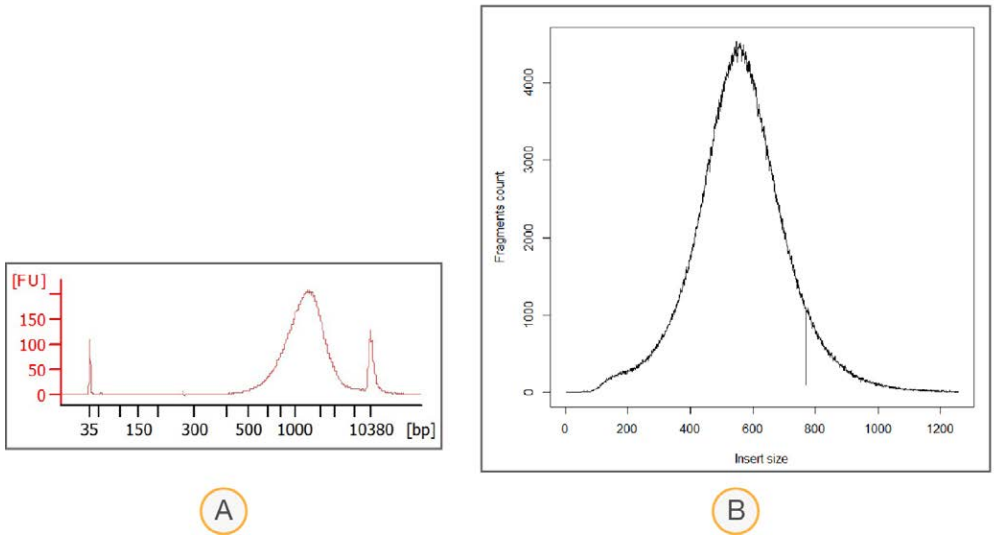
When performing quality control on TruSeq DNA PCR-Free Library Prep libraries, PCR-Free library fragment sizes measured on the Bioanalyzer are substantially larger than would be predicted or derived from sequencing data. The larger size is due to anomalous migration of fragments on the chip due to the presence of certain structural features, which would normally be removed if a subsequent PCR-enrichment step were performed. Figure 7 and Figure 8 show a comparison between library fragment sizes derived by a Bioanalyzer and the corresponding insert sizes derived from the alignment of paired-end reads to a suitable reference sequence.

Figure 7 Example TruSeq DNA PCR-Free Library Prep 350 bp Insert Library Distribution



A Bioanalyzer
B Paired-End Alignment

Figure 8 Example TruSeq DNA PCR-Free Library Prep 550 bp Insert Library Distribution



A Bioanalyzer
B Paired-End Alignment

Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 2 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-indexed DNA libraries are normalized to 2 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	2 (second plate for pooling only, if pooling ≤ 40 samples)	15°C to 30°C	User
96-well midi plate (for pooling only, if pooling > 40 samples)	1	15°C to 30°C	User
Microseal 'B' adhesive seals	5	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each library to 2 nM	15°C to 30°C	User

Preparation

- Remove the TSP1 plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up ALP*.
  - Let it thaw at room temperature.
  - Centrifuge the thawed plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed plate.
- [For pooling only] Review the *TruSeq Library Prep Pooling Guide (part # 15042173)*.
- Apply a DCT barcode label to a new 96-well HSP plate.
- [For pooling only] Apply a PDP barcode label to a new 96-well HSP plate if pooling ≤ 40 samples or a 96-well midi plate if pooling > 40 samples.

Make DCT

1 Transfer 5 µl of the library from each well of the TSP1 plate to the corresponding well of the new HSP plate labeled with the DCT barcode.

2 Normalize the library concentration in each well to 2 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

NOTE
Depending on the yield quantification data of each library, the final volume in the plate can vary from 5–100 µl.

3. Mix thoroughly as follows:
   a. Seal the plate with a Microseal 'B' adhesive seal.
   b. Shake the plate on a microplate shaker at 1000 rpm for 2 minutes.
4. Centrifuge the plate at 280 × g for 1 minute.
5. Remove the adhesive seal from the plate.
6. Do the following, depending on the type of library you want to generate:
   - For libraries that are not pooled, the protocol stops here. Do the following:
     - To continue immediately to sequencing, proceed to cluster generation. For more information, see the cluster generation section of the system guide for your Illumina platform.
     - To stop here before sequencing, seal the DCT plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.
   - For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)

NOTE
Do not make a PDP plate if you are not pooling samples.

1. Determine the number of samples to be combined together for each pool.

   NOTE
   Avoid pooling 2 samples with the same index.

2. Do the following:
   - If pooling 2–24 samples:
     - Transfer 5 µl of each normalized library to be pooled from the DCT plate to 1 well of the new HSP plate labeled with the PDP barcode.
     - The total volume in each well is 5X the number of combined sample libraries and 10–120 µl (2–24 libraries). For example, the volume for 2 samples is 10 µl, the volume for 12 samples is 60 µl, or the volume for 24 samples is 120 µl.
   - If pooling 25–96 samples:
     - Using a multichannel pipette, transfer 5 µl of each normalized library in column 1 of the DCT plate to column 1 of the new HSP or midi plate labeled with the PDP barcode.
     - Transfer 5 µl of each normalized library in column 2 of the DCT plate to column 1 of the PDP plate.
     - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
       - Seal the plate with a Microseal 'B' adhesive seal.
       - Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.
     - Centrifuge the plate at 280 × g for 1 minute.
     - Remove the adhesive seal from the plate.
     - Combine the contents of each well of column 1 into well A2 of the plate for the final pool.
3. Mix thoroughly as follows:
   a. Seal the plate with a Microseal 'B' adhesive seal.
   b. Shake the plate on a microplate shaker at 1800 rpm for 2 minutes.
4. Centrifuge the plate at 280 × g for 1 minute.

5 Do the following:

- To continue immediately to sequencing, proceed to cluster generation. For more information, see the system guide for your Illumina sequencing platform.
- To stop here before sequencing, store the sealed PDP plate at -25°C to -15°C.

Supporting Information

Appendix A

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Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

Acronyms

Table 20 TruSeq DNA PCR-Free Library Prep Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CEP	Clean Up End Repair Plate
CFP	Covaris Fragmentation Plate
CSP	Clean Up Sheared DNA Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligation Control
DAP	DNA Adapter Plate
DCT	Diluted Cluster Template Plate
DNA	Customer Sample DNA Plate
dsDNA	double-stranded DNA
ERP2	End Repair Mix 2
EUC	Experienced User Card
gDNA	genomic DNA
HS	High Sample
HSP	Hard-Shell Plate
HT	High Throughput
IEM	Illumina Experiment Manager
IMP	Insert Modification Plate
LIG2	Ligation Mix 2
LS	Low Sample
LT	Low Throughput
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate

Acronym	Definition
RSB	Resuspension Buffer
SPB	Sample Purification Beads
STL	Stop Ligation Buffer
TSP1	Target Sample Plate 1

Kit Contents

Check to make sure that you have all of the reagents identified in this section before starting the TruSeq DNA PCR-Free Library Prep protocol.

The TruSeq DNA PCR-Free LT Library Prep Kit is available in a Set A and a Set B. Each TruSeq DNA PCR-Free LT Library Prep Kit contains enough reagents to prepare up to 24 samples. When used together, sets A and B allow for pooling up to 24 samples using the 12 different indexes in each kit.

Table 21 TruSeq DNA PCR-Free Library Prep Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indexes
TruSeq DNA PCR-Free LT Library Prep Kit - Set A	FC-121-3001	24	12
TruSeq DNA PCR-Free LT Library Prep Kit - Set B	FC-121-3002	24	12
TruSeq DNA PCR-Free HT Library Prep Kit	FC-121-3003	96	96

TruSeq DNA PCR-Free LT Library Prep Kit

The TruSeq DNA PCR-Free LT Library Prep Kit contains 2 boxes: a Set A or Set B box and an SP Beads box.

24 Samples - Set A or Set B Box

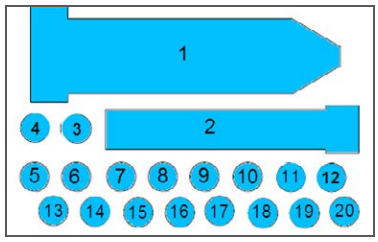
You receive either box A or B with the kit depending on the set you ordered. These boxes also contain plate barcode labels.

Store at -25°C to -15°C

These boxes are shipped on dry ice. As soon as you receive them, store the following components at -25°C to -15°C.

Set A

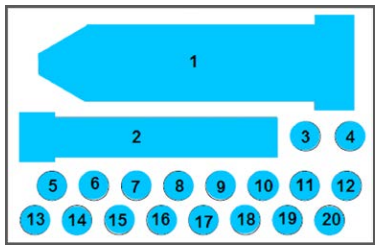
Figure 9 TruSeq DNA PCR-Free LT Library Prep Kit, 24 Samples-Set A (Box 1 of 2), part # 15037063



Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP2	15036418	End Repair Mix 2
3	ATL	15012495	A-Tailing Mix
4	LIG2	15036183	Ligation Mix 2
5	CTE	15026774	End Repair Control
6	CTA	15026775	A-Tailing Control
7	CTL	15026776	Ligation Control
8	STL	15012546	Stop Ligation Buffer
9	AD002	15026621	DNA Adapter Index 2
10	AD004	15026623	DNA Adapter Index 4
11	AD005	15026624	DNA Adapter Index 5
12	AD006	15026625	DNA Adapter Index 6
13	AD007	15026627	DNA Adapter Index 7
14	AD012	15026632	DNA Adapter Index 12
15	AD013	15024641	DNA Adapter Index 13
16	AD014	15024642	DNA Adapter Index 14
17	AD015	15024643	DNA Adapter Index 15
18	AD016	15024644	DNA Adapter Index 16
19	AD018	15024646	DNA Adapter Index 18
20	AD019	15024647	DNA Adapter Index 19

Set B

Figure 10 TruSeq DNA PCR-Free LT Library Prep Kit, 24 Samples-Set B (Box 1 of 2), part # 15037061



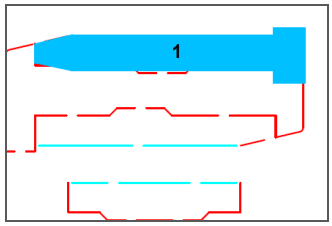
Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP2	15036418	End Repair Mix 2
3	ATL	15012495	A-Tailing Mix
4	LIG2	15036183	Ligation Mix 2
5	CTE	15026774	End Repair Control
6	CTA	15026775	A-Tailing Control
7	CTL	15026776	Ligation Control
8	STL	15012546	Stop Ligation Buffer
9	AD001	15026620	DNA Adapter Index 1
10	AD003	15026622	DNA Adapter Index 3
11	AD008	15026628	DNA Adapter Index 8
12	AD009	15026629	DNA Adapter Index 9
13	AD010	15026630	DNA Adapter Index 10
14	AD011	15026631	DNA Adapter Index 11
15	AD020	15024648	DNA Adapter Index 20
16	AD021	15024649	DNA Adapter Index 21
17	AD022	15024650	DNA Adapter Index 22
18	AD023	15024651	DNA Adapter Index 23
19	AD025	15024653	DNA Adapter Index 25
20	AD027	15024654	DNA Adapter Index 27

24 Samples - SP Beads Box

Store at 2°C to 8°C

This box is shipped at 2°C to 8°C. As soon as you receive it, store the components at 2°C to 8°C.

Figure 11 TruSeq DNA PCR-Free LT Library Prep Kit, 24 Samples SP Beads (Box 2 of 2), part # 15037158



Slot	Reagent	Part #	Description
1	SPB	15037172	Sample Purification Beads

TruSeq DNA PCR-Free HT Library Prep Kit

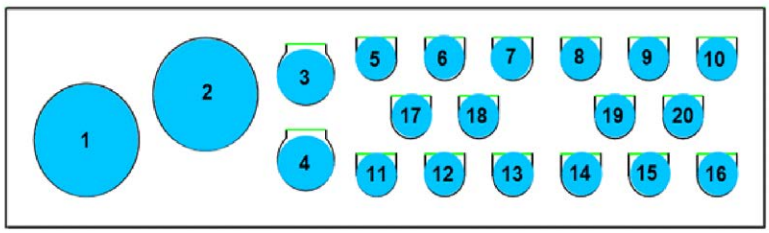
The TruSeq DNA PCR-Free HT Library Prep Kit contains 3 boxes: a core reagent box, an Adapter Plate box, and an SP Beads box.

96 Samples - Core Reagents Box

Store at -25°C to -15°C

This box is shipped on dry ice. As soon as you receive it, store the following components at -25°C to -15°C. This box also contains plate barcode labels.

Figure 12 TruSeq DNA PCR-Free HT Library Prep Kit, 96 Samples (Box 1 of 2), part # 15037059



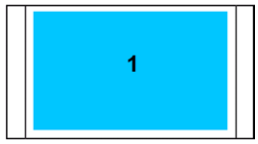
Slot	Reagent	Part #	Description
1–2	RSB	15026770	Resuspension Buffer
3–4	ERP2	15036182	End Repair Mix 2
5–6	ATL	15012495	A-Tailing Mix
7–8	LIG2	15036184	Ligation Mix 2
9–10	CTE	15026774	End Repair Control
11–12	CTA	15026775	A-Tailing Control
13–14	CTL	15026776	Ligation Control
15–16	STL	15012546	Stop Ligation Buffer

96 Samples - Adapter Plate Box

Store at -25°C to -15°C

This box is shipped on dry ice. As soon as you receive it, store the contents at -25°C to -15°C.

Figure 13 TruSeq DNA PCR-Free HT Library Prep Kit, 96, Adapter Plate Box, part # 15032317



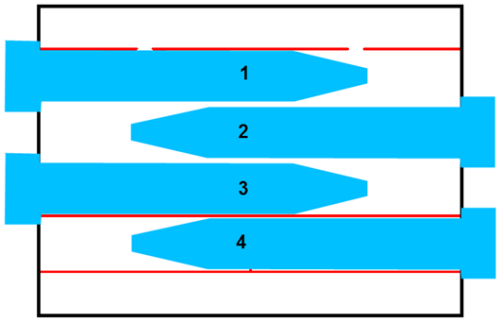
Slot	Reagent	Part #	Description
1	DAP	15016426	DNA Adapter Plate, 96plex

96 Samples - SP Beads Box

Store at 2°C to 8°C

This box is shipped at 2°C to 8°C. As soon as you receive it, store the components at 2°C to 8°C.

Figure 14 TruSeq DNA PCR-Free HT Library Prep Kit, 96 Samples SP Beads (Box 2 of 2), part # 15037163



Slot	Reagent	Part #	Description
1–4	SPB	15037172	Sample Purification Beads

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before starting the TruSeq DNA PCR-Free Library Prep protocol. The requirement for some supplies is dependent upon the protocol performed (LS or HS) and these items are specified in separate tables.

NOTE
The TruSeq DNA PCR-Free Library Prep protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Table 22 User-Supplied Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
High Sensitivity DNA Kit	Agilent Technologies, part # 5067-4626
Ice bucket	General lab supplier
KAPA Library Quantification Kit - Illumina/Universal	KAPA Biosystems, part # KK4824
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001

Consumable	Supplier
microTUBE AFA Fiber 6x16mm with • Crimp-Cap or • Pre-Slit Snap-Cap (for use with Covaris M220)	Covaris, part # • 520052 or • 520045
PCR grade water	General lab supplier
Qubit assay tubes or Axygen PCR-05-C tubes	Life Technologies, catalog # Q32856 or VWR, part # 10011-830
Qubit dsDNA BR Assay Kit	Life Technologies, catalog # • 100 assays, Q32850 • 500 assays, Q32853
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free 8-tubes strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
Tween 20	Sigma-Aldrich, part # P7949
Ultra pure water	General lab supplier

Table 23 User-Supplied Consumables - Additional Items for LS Processing

Consumable	Supplier
96-well 0.3 ml skirtless PCR plates or Twin.tec 96-well PCR plates	E&K Scientific, part # 480096 or Eppendorf, part # 951020303

Table 24 User-Supplied Consumables - Additional Items for HS Processing

Consumable	Supplier
96-well storage plates, round well, 0.8 ml (“midi” plate)	Fisher Scientific, part # AB-0859
Hard-Shell 96-well PCR Plates (“HSP” plate)	Bio-Rad, part # HSP-9601

Table 25 User-Supplied Equipment

Equipment	Supplier
2100 Bioanalyzer Desktop System	Agilent Technologies, part # G2940CA

Equipment	Supplier
One of the following Covaris systems: • S2 • S220 • E210 • M220	Covaris M220, part # 500295 For all other models, contact Covaris
Magnetic stand-96	Life Technologies, catalog # AM10027
Microplate centrifuge	General lab supplier
Qubit 2.0 Fluorometer	Life Technologies, catalog # Q32866
Vortexer	General lab supplier
qPCR system See *qPCR Systems* on page 75.	General lab supplier

Table 26 User-Supplied Equipment - Additional Items for LS Processing

Equipment	Supplier
96-well thermal cycler (with heated lid)	General lab supplier

Table 27 User-Supplied Equipment - Additional Items for HS Processing

Equipment	Supplier
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V) or • 14216-214 (230 V)
Midi plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier
SciGene TruTemp Heating System Note: Two systems are recommended to support successive heating procedures.	Illumina, catalog # • SC-60-503 (115 V) or • SC-60-504 (220 V)

qPCR Systems

The following table lists the validated qPCR systems for the TruSeq DNA PCR-Free Library Prep protocol.

Equipment	Supplier
CFX96 Touch Real-Time PCR Detection System[1]	Bio-Rad, part # 185-5195
Mx3000P qPCR System	Agilent, part # 401511

1. Illumina recommends using CFX Manager software version 3.0 with Cq Determination mode: Single Threshold; Baseline Setting:Baseline Subtracted Curve Fit and Apply Fluorescent Drift Correction for data analysis. This setting can correct for abnormalities in fluorescence intensity of the standard curve caused by the instrument. For software installation, contact Bio-Rad.

Indexed Adapter Sequences

This section details the indexed adapter sequences.

TruSeq DNA PCR-Free LT Library Prep Kit Indexed Adapter Sequences

The TruSeq DNA PCR-Free LT Library Prep Kit contains the following indexed adapter sequences.

NOTE

- The index numbering is not contiguous. There is no Index 17, 24, or 26.
- The base in parentheses () indicates the base for the seventh cycle and is not considered as part of the index sequence. Record the index in the sample sheet as only 6 bases. For indexes 13 and above, the seventh base (in parentheses) might not be A, which is seen in the seventh cycle of the Index Read.
- For more information on the number of cycles used to sequence the Index Read, see the system guide for your Illumina sequencing platform.

Table 28 TruSeq DNA PCR-Free LT Library Prep Kit Set A Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AD002	CGATGT(A)	AD013	AGTCAA(C)
AD004	TGACCA(A)	AD014	AGTTCC(G)
AD005	ACAGTG(A)	AD015	ATGTCA(G)
AD006	GCCAAT(A)	AD016	CCGTCC(C)
AD007	CAGATC(A)	AD018	GTCCGC(A)
AD012	CTTGTA(A)	AD019	GTGAAA(C)

Table 29 TruSeq DNA PCR-Free LT Library Prep Kit Set B Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AD001	ATCACG(A)	AD020	GTGGCC(T)
AD003	TTAGGC(A)	AD021	GTTTCG(G)
AD008	ACTTGA(A)	AD022	CGTACG(T)
AD009	GATCAG(A)	AD023	GAGTGG(A)
AD010	TAGCTT(A)	AD025	ACTGAT(A)
AD011	GGCTAC(A)	AD027	ATTCCT(T)

TruSeq DNA PCR-Free HT Library Prep Kit Indexed Adapter Sequences

The DAP in the TruSeq DNA PCR-Free HT Library Prep Kit contains the following indexed adapter sequences:

NOTE
The Index recorded in the sample sheet is the full 8 bases and 8 bases are sequenced per indexed read.

Table 30 TruSeq DNA PCR-Free HT Library Prep Kit Indexed Adapter 1 Sequences

Adapter	Sequence		Adapter	Sequence
D701	ATTACTCG		D707	CTGAAGCT
D702	TCCGGAGA		D708	TAATGCGC
D703	CGCTCATT		D709	CGGCTATG
D704	GAGATTCC		D710	TCCGCGAA
D705	ATTCAGAA		D711	TCTCGCGC
D706	GAATTCGT		D712	AGCGATAG

Table 31 TruSeq DNA PCR-Free HT Library Prep Kit Indexed Adapter 2 Sequences

Adapter	Sequence		Adapter	Sequence
D501	TATAGCCT		D505	AGGCGAAG
D502	ATAGAGGC		D506	TAATCTTA
D503	CCTATCCT		D507	CAGGACGT
D504	GGCTCTGA		D508	GTACTGAC

The DAP dual-index layout:

Figure 15 DAP Dual-Indexed Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-D503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 32 Illumina General Contact Information

Address	5200 Illumina Way San Diego, CA 92122 USA
Website	www.illumina.com
Email	techsupport@illumina.com

Table 33 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.html.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to support.illumina.com, select a product, then click **Documentation & Literature**.

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